The transcriptional repressor Snail promotes mammary tumor recurrence

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Summary

Breast cancer recurrence is a fundamental clinical manifestation of tumor progression and represents the principal cause of death from this disease. Using a conditional transgenic mouse model for the recurrence of HER2/neu-induced mammary tumors, we demonstrate that the transcriptional repressor *Snail* is spontaneously upregulated in recurrent tumors in vivo and that recurrence is accompanied by epithelial-to-mesenchymal transition (EMT). Consistent with a causal role for Snail in these processes, we show that Snail is sufficient to induce EMT in primary tumor cells, that Snail is sufficient to promote mammary tumor recurrence in vivo, and that high levels of *Snail* predict decreased relapse-free survival in women with breast cancer. In aggregate, our observations strongly implicate Snail in the process of breast cancer recurrence.

Introduction

Breast cancer is the most common malignancy diagnosed among women worldwide and is the leading cause of cancer mortality (Parkin et al., 2005). In 2005, more than 1.1 million women will be diagnosed with breast cancer, and over 400,000 women will die from this disease. While breast cancer incidence has steadily increased in Western countries, breast cancer mortality rates have declined for more than a decade such that nearly 90% of women diagnosed with breast cancer survive for at least 5 years. As a consequence of its high incidence and favorable prognosis, breast cancer is the most prevalent cancer in the world today (Parkin et al., 2005). Among these women, tumor dormancy followed by recurrence—local, regional, or distant—represents the most common cause of breast cancer mortality.

Although recurrence generally occurs relatively early, between 1 and 2 years after surgery (Demicheli et al., 2004; Saphner et al., 1996), a considerable proportion of breast cancers that appear cured resurface as local or distant tumor recurrences 10 or 20 years after surgery (Fisher et al., 2004; Weiss et al., 2003). This suggests that in many cases tumor cells have already disseminated to distant sites by the time that breast cancers are diagnosed. Indeed, analysis of bone marrow specimens indicates that residual cancer cells are detectable in up to 40% of primary breast cancer patients who lack clinical or histopathological signs of metastasis (Pantel et al., 2003). These and other observations argue that breast cancer recurrence represents a major obstacle to curing this disease.

Despite the central role of recurrence in breast cancer mortality, little is known about the cellular or molecular events responsible. A limited number of clinical and molecular characteristics of breast cancers have been shown to correlate with relapse-free survival. In women with breast cancer, the most important factors for predicting recurrence are tumor size and the extent of lymph node involvement (Carter et al., 1989; Valagussa et al., 1978). In addition, a number of molecular markers for aggressive tumor behavior can be used to identify breast cancer patients at high risk for recurrence, including HER2/neu expression, c-*myc* amplification, and estrogen receptor (ER) negativity (Esteva and Hortobagyi, 2004; Schlotter et al., 2003). However, neither these nor other molecular prognostic markers

SIGNIFICANCE

Breast cancer is the most common malignancy diagnosed among women worldwide and is the leading cause of cancer mortality. Among the more than 5 million women currently living with a diagnosis of breast cancer, recurrence represents the most common cause of death from this disease. Nevertheless, while recurrence constitutes a problem of unrivaled clinical importance, little is known about the mechanisms underlying it. Our findings demonstrate that Snail is sufficient to promote mammary tumor recurrence in mice and that its expression is associated with rapid tumor recurrence in women independently of established prognostic factors. These observations provide in vivo genetic evidence for a molecular pathway contributing to mammary tumor recurrence and suggest that Snail may represent a target for cancer therapy.

have been shown to play a causal role in breast cancer recurrence. As such, their mechanistic relationship to this process remains speculative.

Elucidating the molecular mechanisms that allow tumors to evade primary therapy and recur is a critical goal of breast cancer research. In particular, understanding the biology of tumor latency and recurrence would permit improvements in the prediction, prevention, and treatment of breast cancer recurrence. Achieving this goal, however, has been hampered by the lack of animal models that faithfully recapitulate this fundamental step in breast cancer progression. Such models are essential for the rational development and testing of therapeutics targeted against the residual population of neoplastic cells responsible for the majority of breast cancer deaths.

Further compounding difficulties in understanding recurrence is the limited availability of clinical material for analysis. While molecular profiles for primary human breast cancers have become widely available, no comprehensive molecular analysis of recurrent human breast cancers currently exists. Consequently, not only do we lack information on pathways causally involved in recurrence, but we also lack a basic understanding of the specific molecular features that distinguish recurrent breast cancers from the primary tumors from which they arose.

One molecular prognostic marker for poor clinical outcome in breast cancer patients is the proto-oncogene *HER2/neu*. Amplification and overexpression of this receptor tyrosine kinase occurs in 15%–30% of primary human breast cancers and is associated with aggressive tumor behavior, high rates of relapse, and poor prognosis (Berger et al., 1988; Slamon et al., 1987). In recent years trastuzumab (Herceptin), a neutralizing antibody that inhibits the activity of HER2/neu, has been tested in clinical trials for patients with *HER2/neu*-amplified breast cancers. The efficacy of this agent in slowing disease progression and prolonging survival, even in advanced stages of disease, has been demonstrated in multiple studies (Baselga et al., 1996; Slamon et al., 2001; Vogel et al., 2002; Wang et al., 2001). However, even in cases in which trastuzumab is combined with standard chemotherapeutic regimens, breast cancers typically become resistant to therapy and recur (Hortobagyi, 2001). As with cancer recurrence in general, the mechanisms by which *HER2/neu*-amplified breast tumor cells evade the blockade of this pathway are poorly understood.

Given the importance of the proto-oncogene *HER2/neu* in human breast cancers, we have used a mammary-specific, conditional transgenic model to investigate the effects of neu pathway downregulation on the regression and recurrence of mammary tumors induced by this oncogene. Specifically, we have used the tetracycline-regulatory system to inducibly express an activated form of neu in the mammary epithelium of transgenic mice (Moody et al., 2002). When treated with doxycycline, these mice develop multiple invasive mammary adenocarcinomas that regress to a nonpalpable state upon targeted downregulation of the neu pathway. However, consistent with the behavior of human malignancies, the vast majority of mice harboring fully regressed tumors ultimately develop recurrences in the absence of doxycycline treatment and neu expression. As such, this model recapitulates key features of the natural history of human breast cancers relevant to tumor recurrence.

We have employed this conditional transgenic mouse model to investigate secondary pathways involved in breast cancer progression and escape. We now report the identification of a molecular pathway involved in spontaneous mammary cancer recurrence. We show that recurrent neu-induced mammary tumors display phenotypic and molecular alterations characteristic of the epithelial-to-mesenchymal transition (EMT) and spontaneously upregulate expression of *Snail*, a transcriptional repressor previously implicated in EMT. We further show that Snail is sufficient both to induce EMT in neu-induced primary tumor cells and to promote rapid tumor recurrence in vivo following downregulation of the neu pathway.

Finally, analysis of four independent microarray expression data sets derived from prospectively harvested human breast cancer samples revealed that high levels of *Snail* expression strongly predict decreased relapse-free survival in women with breast cancer independently of most currently used prognostic indicators for breast cancer. These studies identify a molecular pathway involved in mammary tumor recurrence in vivo and suggest that Snail may play a role in the progression of human breast cancers.

Results

Fully regressed neu-induced tumors recur spontaneously following a period of dormancy

We previously generated a doxycycline-inducible bitransgenic mouse model for HER2/neu-induced mammary carcinogenesis, referred to as MMTV-rtTA/TetO-NeuNT (MTB/TAN) (Moody et al., 2002). When expression of this activated form of neu is induced with doxycycline, MTB/TAN mice develop multiple invasive mammary adenocarcinomas, many of which metastasize to the lung (Moody et al., 2002). Downregulation of neu expression in the vast majority of these fully formed mammary tumors results in their regression to a nonpalpable state. However, following a latent period, some mice bearing fully regressed tumors develop spontaneous tumor recurrences in the absence of neu expression.

To investigate the mechanisms responsible for the recurrence of neu-induced mammary tumors, we first generated a large cohort of tumor-bearing mice. Of 507 neu-induced primary mammary tumors monitored in 62 MTB/TAN mice, 493 (97%) regressed to a nonpalpable state following doxycycline withdrawal and downregulation of the neu pathway. Following doxycycline withdrawal, two neu-induced primary tumors failed to regress, whereas an additional 11 primary tumors regressed partially and then rapidly resumed neu-independent growth. Thus, in nearly all cases the vast majority of cells within neu-induced primary tumors remain dependent upon neu for maintenance of the transformed state.

Fifty mice in which all tumors had regressed to a nonpalpable state following doxycycline withdrawal were monitored for extended periods of time in the absence of doxycycline. Over a 1 year period, 43 (86%) of these mice spontaneously developed recurrent tumors with a mean latency of 117 days (Figure 1A). Formally, doxycycline-independent tumors arising in mice that had previously harbored mammary tumors could represent either genuine recurrences of neu-initiated tumors or the de novo formation of tumors in the absence of doxycycline. Our repeated observations that uninduced MTB/TAN animals do not develop tumors, even over periods exceeding 18 months, and that recurrent tumors always appear at a site at which a pri-

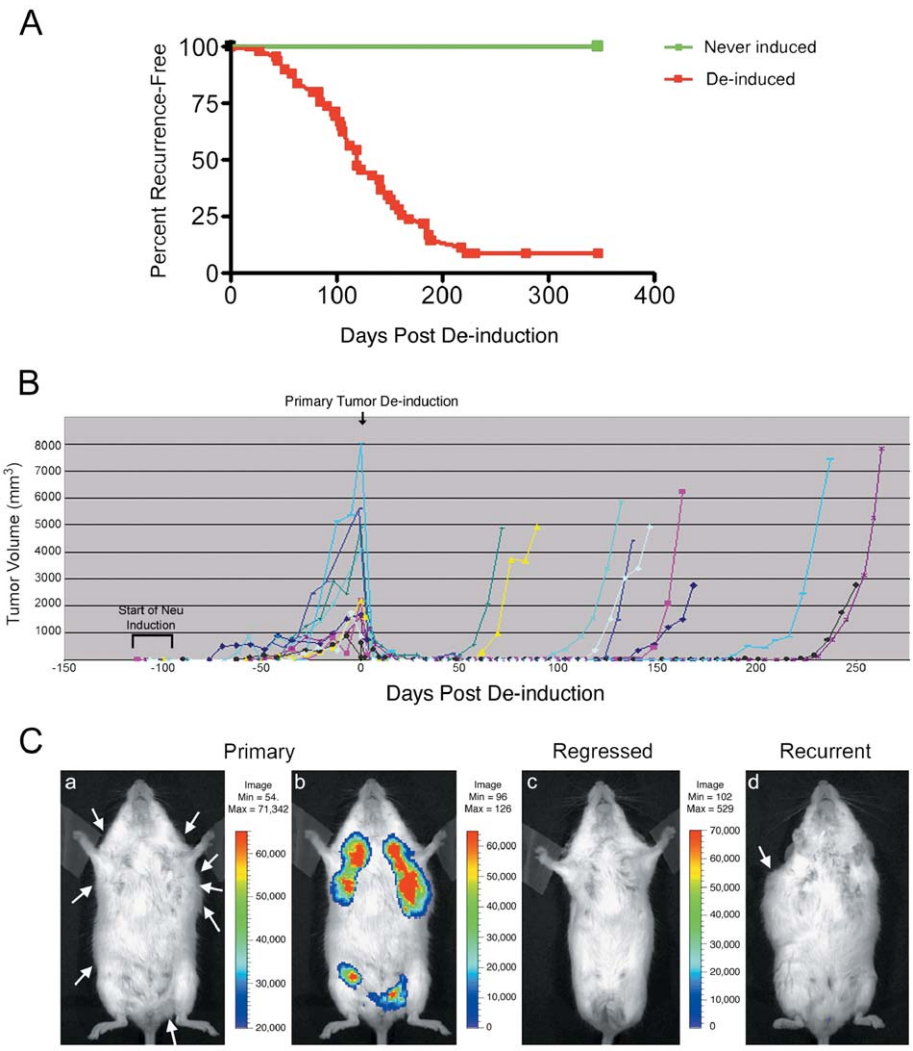

Figure 1. Fully regressed neu-induced tumors recur spontaneously following a latent period

A: Recurrence-free survival for never-induced MTB/TAN mice (n = 13) and MTB/TAN mice deinduced after developing primary tumors on doxycycline (n = 62) (SD 48, range 27–222).

B: Timing of tumor regression and recurrence for individual neu-induced tumors. The time of doxycycline withdrawal (day 0) from tumor-bearing mice is indicated by an arrow. A bracket indicates the range of times (relative to doxycycline withdrawal) at which neu was initially induced by doxycycline treatment.

C: Luciferase imaging of tumor-bearing MTB/TAN mice demonstrating that recurrent tumors do not express the *TAN* transgene in the absence of doxycycline. A mouse with multiple primary tumors (arrows) on doxycycline is shown with (**B**) and without (**A**) luciferase activity overlay. The same mouse after full regression of tumors off doxycycline is shown with luciferase activity overlay (**C**). A second mouse with a recurrent tumor in mammary gland 3R (arrow) lacking luciferase activity is shown in **D**.

mary tumor had previously existed, suggest that these doxycycline-independent tumors represent bona fide recurrences.

To confirm that doxycycline-independent recurrent tumors arise from cells within the original primary tumor, we implanted small fragments of primary tumors from MTB/TAN mice onto the flanks of wild-type mice. Grafted hosts were initially maintained on doxycycline to permit tumor outgrowth, after which time doxycycline was withdrawn. Similar to the behavior of primary mammary tumors in MTB/TAN mice, grafted tumors regressed to a nonpalpable state following doxycycline withdrawal, and a subset of these regressed tumor grafts eventually recurred at the original site following a latent period. Since tumors in nontransgenic hosts could only arise from grafted cells, these data confirm that doxycycline-independent tumors that arise in MTB/TAN mice harboring fully regressed tumors are in fact recurrences derived from cells within the primary tumor. This conclusion is further strengthened by our subsequent demonstration that epithelial cell cultures derived from primary neu-induced mammary tumors also give rise to recurrences when subjected to a similar grafting protocol (see below).

Analysis of mammary tumor behavior in intact MTB/TAN mice revealed that recurrent tumors arose stochastically over an 8 month period, frequently following a prolonged latency period (Figure 1B). The concept of tumor latency in breast cancer patients arises in part from the inability of continuous growth models to explain the kinetics of tumor recurrence in patients, as well as the lack of a relationship between the length of time from surgery to tumor recurrence and the growth rate of tumors once they reappear. Analogous to the situation in humans, the latency for tumor recurrence in MTB/TAN mice was considerably longer than that observed for primary tumor development following neu induction (mean latency, 117 versus 42 days, respectively; Figure 1A and Moody et al., 2002). Similarly, the growth rate of recurrent tumors once they reappeared was unrelated to the length of time between tumor regression and tumor recurrence (Figure 1B). Thus, as with breast cancer patients, the timing of tumor recurrence and the kinetics of recurrent tumor growth cannot be explained by a model postulating constant growth of residual tumor cells. In aggregate, our observations strongly suggest that deinduced MTB/TAN mice harbor residual neoplastic cells at the sites of their

original tumors that persist in a latent state for variable periods of time before reemerging as recurrent disease.

Fully regressed neu-induced tumors recur in the absence of neu expression

In theory, doxycycline-independent tumor recurrences could result from doxycycline-independent activation of the *NeuNT* transgene, compensatory upregulation of endogenous ErbB2, or activation of neu-independent growth and/or survival pathways. Since the *TetO-NeuNT* transgene contains a bicistronic IRES-firefly luciferase cassette, transgene expression can be monitored longitudinally in mice by noninvasive in vivo imaging of luciferase. As predicted, luciferase activity was readily visualized within primary tumors in MTB/TAN mice maintained on doxycycline, whereas mice maintained off doxycycline harboring either fully regressed or recurrent tumors did not express detectable luciferase activity (Figure 1C). In addition, Northern analysis as well as anti-ErbB2 immunohistochemistry failed to detect upregulation of endogenous ErbB2 in recurrent tumors (Figure 2C and data not shown). These observations indicate that spontaneous recurrence of neu-induced mammary tumors occurs by a process other than the doxycycline-independent reactivation of the *NeuNT* transgene or compensatory upregulation of endogenous ErbB2.

Taken together, these results suggest that a subset of cells in neu-induced primary tumors are able to progress to a state that is independent of neu overexpression for survival and growth. Moreover, the fact that MTB/TAN mice bearing fully regressed tumors typically relapse following a latency of up to 8 months indicates that these mice harbor viable residual neoplastic cells that persist in the mammary gland for extended periods of time.

Recurrent tumors display characteristics of EMT

Neu-induced mammary adenocarcinomas in MTB/TAN mice maintained on doxycycline display a characteristic epithelial morphology and stain positively for the luminal epithelial marker cytokeratin 8 (CK8) (Moody et al., 2002). Since mammary tumors induced in mice by different oncogenic pathways have been shown to exhibit distinct histopathological "signatures," we reasoned that doxycycline-independent tumor recurrences that no longer expressed neu might not exhibit the classic neu phenotype (Cardiff et al., 1991). In agreement with this prediction, histopathological examination of multiple neu-negative, doxycycline-independent recurrent tumors revealed that the vast majority of these tumors were composed of spindle-shaped cells with mesenchymal morphology (Figure 2A). Furthermore, immunohistochemical analysis demonstrated that recurrent tumors had downregulated CK8, although occasional CK8-positive cells were detected (Figure 2A).

These findings led us to hypothesize that neu-induced mammary tumors undergo EMT prior to their reemergence as neu-independent recurrent tumors. Consistent with this hypothesis, Northern analysis confirmed the absence of *NeuNT* transgene expression in recurrent tumors and further revealed that expression of the mesenchymal markers *vimentin* and *fibronectin* were upregulated (Figure 2C). Conversely, a striking reduction in expression of the epithelial marker *E-cadherin* was observed in virtually every neu-independent recurrent tumor (Figure 2C). E-cadherin is an essential component of adherens junctions in epithelial cells, and its expression is commonly lost in EMT. Immunofluorescence studies confirmed that recurrent tumors downregulate expression of CK8 and E-cadherin and upregulate expression of the mesenchymal marker S100A4 (fibroblast-specific protein [Fsp1]) (Figure 2D). These findings demonstrate that recurrent tumors display multiple features characteristic of cells that have undergone EMT.

Mesenchymal recurrences arise from primary epithelial tumors

Since primary neu-dependent mammary tumors and doxycycline-independent tumor recurrences exhibit strikingly different morphological phenotypes, we wished to confirm that mesenchymal recurrences arise from primary epithelial tumors. Two lines of evidence indicate that this is indeed the case. First, we performed both Northern and histological analysis on the small number of neu-induced primary mammary tumors that regressed only partially following doxycycline withdrawal before resuming growth. This analysis revealed that incompletely regressing doxycycline-independent primary tumors, which remain clinically apparent throughout their period of partial regression and regrowth, display the same spindle cell phenotype and identical molecular characteristics with respect to markers of EMT as doxycycline-independent recurrences (Figure 2C and data not shown). This suggests that the same cellular processes that lead to tumor recurrence also contribute to the development of neu-independent primary tumors.

Second, primary MTB/TAN tumors grafted onto the flanks of wild-type mice were allowed to engraft and grow on doxycycline before being induced to regress to a nonpalpable state by withdrawal of doxycycline treatment. Grafted mice bearing fully regressed tumors were monitored for spontaneous recurrence in the absence of doxycycline. Primary tumor grafts were biopsied prior to doxycycline withdrawal, and doxycycline-independent tumors that eventually recurred at graft sites were harvested. Histological analysis of these matched sets of primary and recurrent tumor grafts demonstrated that primary tumor grafts displayed the classic neu epithelial phenotype, whereas doxycycline-independent recurrences that arose at the same sites had acquired a mesenchymal phenotype (Figure 2B). Taken together, these two lines of evidence indicate that mesenchymal-appearing, doxycycline-independent tumors that arise at the site of fully regressed neu-induced neoplasms constitute recurrences of epithelial tumors that have undergone EMT.

***Snail*, an inducer of EMT, is spontaneously upregulated in recurrent mammary tumors**

A major regulator of EMT during embryonic mesoderm and neural crest development is the zinc finger transcription factor *Snail*. First identified in *Drosophila* mutant embryos exhibiting defective mesoderm invagination, Snail is required for normal mesoderm development in mice (Grau et al., 1984; Carver et al., 2001). In addition, Snail directly represses *E-cadherin* transcription in both mouse and human epithelial cell lines (Batlle et al., 2000; Cano et al., 2000). Similarly, both Snail and its closely related family member Slug can repress endogenous *E-cadherin* expression in human breast cancer cell lines (Hajra et al., 2002). Consistent with this, invasive ductal carcinomas of the breast have been reported to express *Snail* in a manner that is inversely correlated with *E-cadherin* expression (Cheng et al., 2001).

In light of the association of Snail with EMT, we wished to

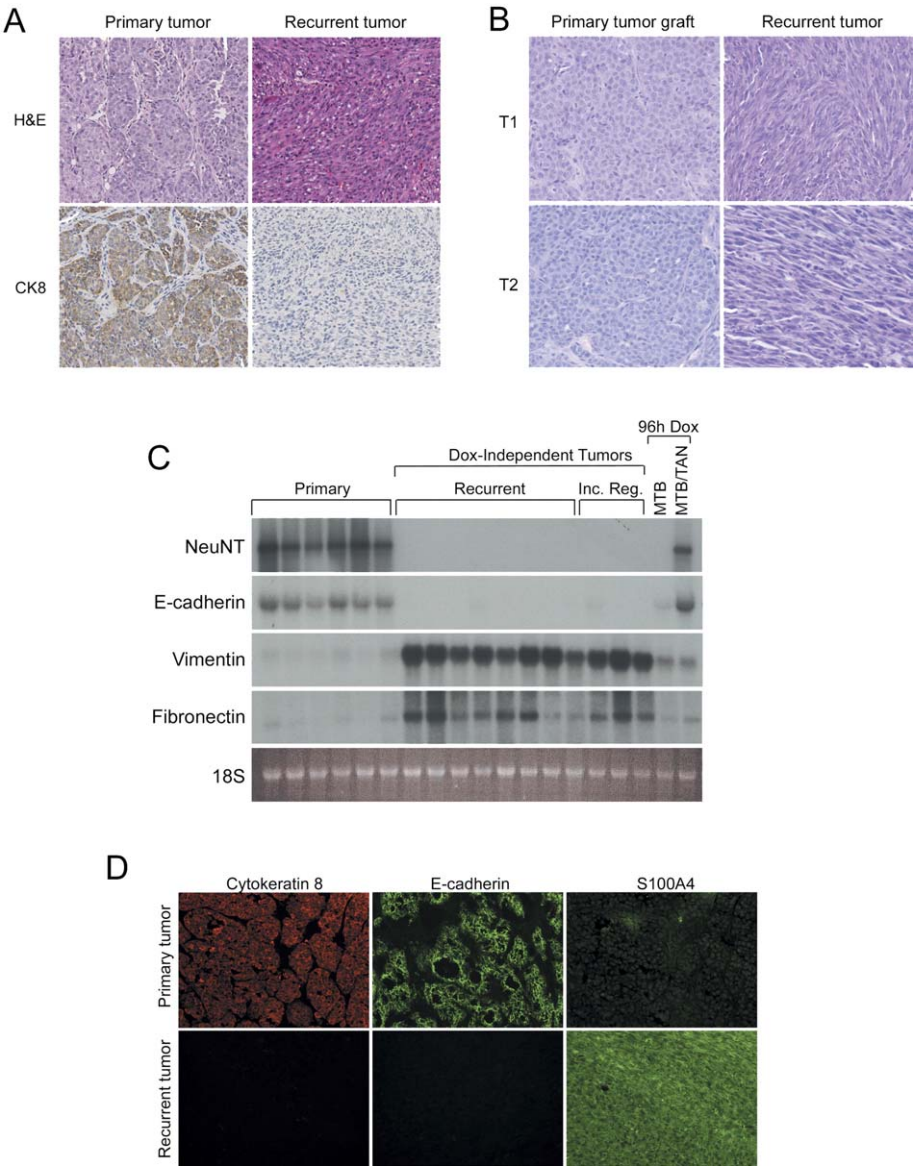

Figure 2. Recurrent tumors display characteristics of epithelial-to-mesenchymal transition

A: H&E- and anti-CK8-stained sections from representative MTB/TAN primary (neu-expressing) and MTB/TAN recurrent (neu-negative) tumors demonstrating the transition from epithelial to mesenchymal morphology and marker expression. Magnification, 400×.

B: H&E-stained sections of two primary tumor grafts allowed to engraft on doxycycline and biopsied while on doxycycline. Tumors either partially regressed and then resumed growth in the absence of doxycycline (T1) or completely regressed and recurred at the same site (T2). Magnification, 400×.

C: Northern analysis of primary and recurrent MTB/TAN tumors for expression of epithelial and mesenchymal markers. rRNA (18S) is shown as a loading control.

D: Immunofluorescence (IF) analysis of CK8, E-cadherin, and S100A4 expression in representative primary and recurrent tumors. Magnification, 400×.

determine whether *Snail* was upregulated in recurrent, neu-induced mammary tumors that exhibited features of EMT. Microarray analysis of doxycycline-independent MTB/TAN tumors demonstrated a 9-fold increase in *Snail* expression compared to primary MTB/TAN tumors ($p = 8 \times 10^{-6}$), whereas only a 1.8-fold change in expression was detected in its closely related family member, *Slug* ($p = 10^{-4}$) (Figure 3A). Northern analysis confirmed that recurrent tumors express increased levels of *Snail* compared to primary tumors (Figure 3B). Similarly, consistent with their spindle cell appearance, primary mammary tumors that regressed only partially prior to resuming growth in a neu- and doxycycline-independent manner also displayed increased levels of *Snail* (Figure 3B).

Snail induces EMT in primary neu-induced tumor cells in vitro

Since we had observed a correlation between Snail expression and acquisition of a mesenchymal phenotype in doxycycline-independent tumors, we wished to test whether Snail could induce EMT in primary neu-induced mammary tumor cells. Although several studies in epithelial cell lines have demonstrated that Snail expression can induce EMT, some normal and malignant epithelial cell lines have proven to be resistant to this phenomenon in vitro (Batlle et al., 2000; Cano et al., 2000; Zhou et al., 2004). To determine whether Snail is capable of inducing EMT in neu-expressing tumor cells, we transduced primary tumor cells maintained on doxycycline with a retroviral vector encoding Snail or with the control retroviral vector pk1. Infected cells were selected in puromycin-containing media, and immunoblotting was performed to assess Snail expression in transduced cells (Figure 3C). Within two weeks of transduction, pk1-Snail-infected cells acquired a fibroblastic spindle cell phenotype accompanied by loss of cell-to-cell contacts (Figure 3D). In contrast, pk1-infected control cells retained their epithelial morphology. These results demonstrate that Snail expres-

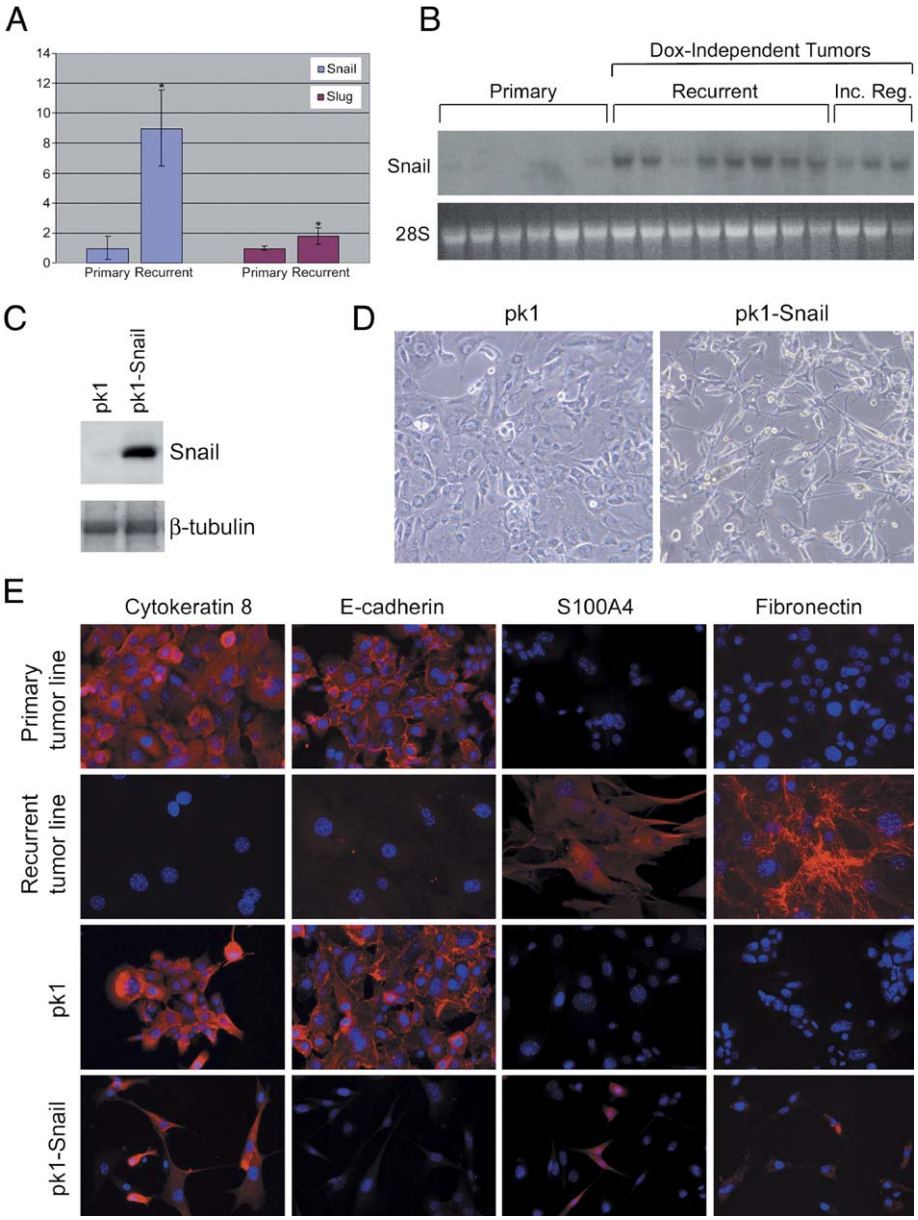

Figure 3. *Snail* is spontaneously upregulated in recurrent mammary tumors and induces EMT in primary neu-induced tumor cells

A: Microarray analysis of mean *Snail* and *Slug* expression levels in primary and recurrent tumors. Expression levels were significantly different between primary tumors and recurrent tumors for both genes ($p = 8 \times 10^{-6}$ for *Snail*; $p = 0.0001$ for *Slug*). Error bars indicate standard error of the mean.

B: Northern analysis of *Snail* expression in primary, recurrent, and incompletely regressing (Inc. Reg.) tumors. rRNA (28S) is shown as a loading control.

C: Western analysis of Snail expression in pk1- and pk1-Snail-infected primary tumor cells.

D: Photomicrographs of pk1- and pk1-Snail-infected primary tumor cells showing spindle cell morphology of Snail-expressing cells.

E: IF analysis of uninfected primary tumor cells, uninfected recurrent tumor cells, and primary tumor cells infected with pk1 or pk1-Snail. Magnification, 400× (**D and E**).

sion in neu-induced primary tumor cells is sufficient to induce the acquisition of a spindle-shaped morphology.

Since the transition to a spindle-shaped morphology is consistent with EMT, we next asked whether alterations associated with EMT also occurred at the molecular level. Primary tumor cells transduced with pk1- or pk1-Snail-expressing retroviral vectors, as well as nontransduced primary and recurrent tumor cells, were immunostained with antibodies directed against epithelial and mesenchymal markers. As expected, CK8 and E-cadherin were expressed in both uninfected and pk1-infected primary tumor cells, whereas expression of these epithelial markers was lost in cells cultured from recurrent tumors (Figure 3E). Conversely, the mesenchymal markers S100A4 and fibronectin were highly upregulated in the recurrent tumor cell line compared to uninfected or pk1-infected primary tumor cells.

Notably, while complete repression of E-cadherin expression was observed in primary tumor cells transduced with pk1-Snail, CK8 expression was lost in only a fraction of Snail-expressing primary tumor cells (Figure 3E). Similarly, a marked increase in S100A4 and fibronectin expression was evident in a subset of pk1-Snail-infected primary tumor cells. The upregulation of mesenchymal markers in only a subset of Snail-transduced cells contrasts with the ubiquitous upregulation of mesenchymal markers in recurrent tumor cells. This differential behavior may be due to the concurrent expression of neu in tumor cell lines maintained on doxycycline, since neu may impede Snail's ability to induce the full phenotypic manifestations of EMT. Alternately, full manifestation of the EMT phenotype may require additional genetic or epigenetic alterations besides Snail expression. Nonetheless, complete transition to a mesenchymal morphology occurs in pk1-Snail-infected cells. In aggregate, these observations demonstrate that Snail expression triggers

acquisition of a mesenchymal phenotype in neu-induced primary tumor cells.

Snail promotes the recurrence of neu-induced primary tumors

Since virtually all transgene-independent MTB/TAN recurrent tumors display a mesenchymal phenotype and express high levels of *Snail*, we hypothesized that Snail may directly promote tumor recurrence. To investigate this possibility, we grafted pk1-Snail- or pk1-transduced primary tumor cells onto the flanks of nude mice maintained on doxycycline. Both sets of cells formed tumors with equal efficiency. As predicted, tumor grafts derived from pk1-transduced primary tumor cells displayed an epithelial morphology, whereas tumor grafts arising from pk1-Snail-transduced cells exhibited a mesenchymal morphology despite continued expression of the neu oncogene (Figure 4A). To confirm that tumors derived from pk1-Snail-transduced cells do not have altered *NeuNT* transgene expression prior to the withdrawal of doxycycline, we assayed luciferase activity levels in pk1- and pk1-Snail-transduced tumor grafts harvested from mice maintained on doxycycline. As expected, in mice maintained on doxycycline both pk1-Snail and pk1 tumor grafts expressed high, comparable levels of the *NeuNT-IRES-Luciferase* transgene (Figure 4B).

When pk1- and pk1-Snail-transduced tumor grafts reached a size of 27 mm^3, doxycycline was withdrawn. All tumors regressed to a nonpalpable state following doxycycline withdrawal regardless of Snail expression status. Strikingly, within 60 days of neu downregulation, 17 of 20 (85%) pk1-Snail-transduced tumors recurred, whereas only 1 of 20 (5%) pk1-transduced tumors recurred ($p < 0.0001$) (Figure 4C). Moreover, similar to recurrent tumors that arose in tumor-bearing MTB/TAN mice withdrawn from doxycycline, regressed pk1-Snail tumor grafts that recurred in the absence of doxycycline lacked detectable *NeuNT* transgene expression (Figure 4B).

Notably, pk1-Snail-induced recurrent tumors displayed a fibroblastic phenotype similar both to doxycycline-independent tumor recurrences arising in MTB/TAN mice and to pk1-Snail-transduced primary tumor grafts arising in mice maintained on doxycycline (Figure 4A). Consistent with this, immunofluorescence analysis revealed the downregulation of E-cadherin expression and upregulation of S100A4 in both doxycycline-dependent pk1-Snail primary tumor grafts and doxycycline-independent pk1-Snail recurrent tumor grafts (Figure 4D). In contrast, high levels of E-cadherin and low levels of S100A4 were observed in epithelial-appearing pk1 control tumor grafts growing in doxycycline-treated mice. Thus, Snail-induced recurrent tumors are morphologically and molecularly similar to MTB/TAN recurrent tumors that arise in intact mice in the absence of *NeuNT* transgene expression. In aggregate, these data demonstrate that Snail promotes the rapid recurrence of primary tumor cells in vivo following downregulation of the neu pathway.

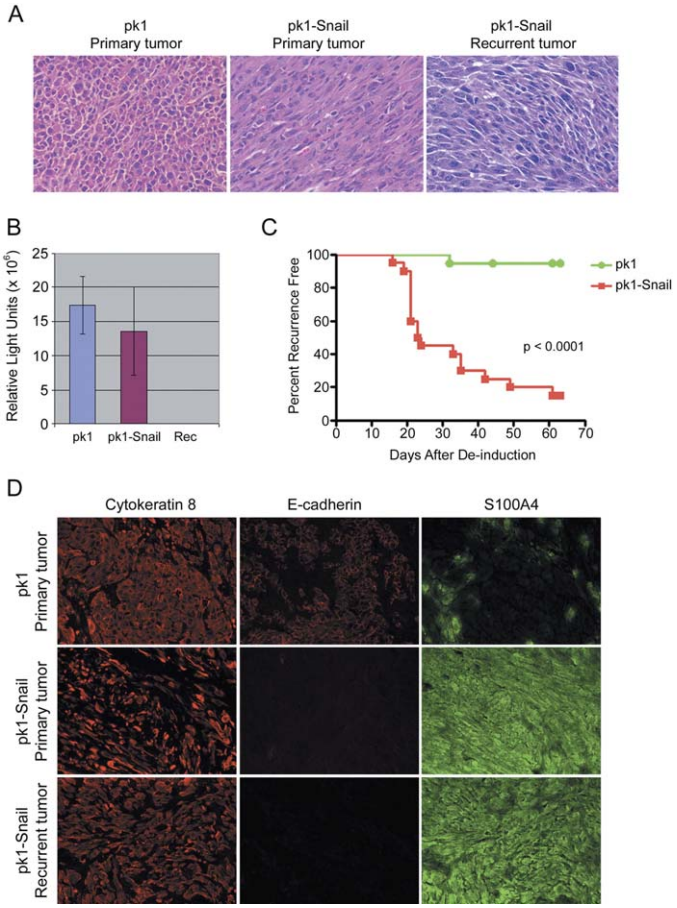

Figure 4. Snail promotes the recurrence of neu-induced primary tumors

A: H&E-stained sections from tumors formed by pk1- and pk1-Snail-infected tumor cells harvested from mice on doxycycline, and from doxycycline-independent, pk1-Snail-transduced recurrent tumors. Magnification, 400×.

B: Luciferase activity levels in pk1 and pk1-Snail tumors in mice on doxycycline, and in pk1-Snail recurrent tumors (Rec) arising after doxycycline withdrawal. n = 6 for each tumor type. Error bars indicate standard error of the mean.

C: Recurrence-free survival for mice engrafted with pk1-infected and pk1-Snail-infected primary tumor cells. Mice were removed from doxycycline on day 0 after the injected tumor cells had formed a tumor ~27 mm^3 in size. Recurrence rates were significantly higher in pk1-Snail-infected cells ($p < 0.0001$).

D: IF analysis of tumors formed by pk1- and pk1-Snail-infected tumor cells harvested from mice on doxycycline, and of doxycycline-independent, pk1-Snail-transduced recurrent tumors. Magnification, 400×.

Snail expression predicts decreased relapse-free survival in women with breast cancer

Our observation that enforced Snail expression in primary tumor cells promotes mammary tumor recurrence in mice raised the question of whether Snail might play a similar role in the recurrence of breast cancers in humans. If this were the case, we reasoned that women with primary breast cancers expressing high levels of Snail might relapse at a faster rate than women whose breast cancers expressed lower levels of Snail. Therefore, we examined four microarray expression data sets derived from the analysis of primary human breast cancers in which both *Snail* expression levels and clinical outcome were reported. For each data set, tumor samples were classified as either high or low Snail expressers based on array hybridization levels (for two-color arrays) or absolute expression analysis p values (for Affymetrix arrays). The relationship between Snail expression level and 5 year relapse-free survival was then determined.

In the first series of samples examined, Sorlie et al. profiled

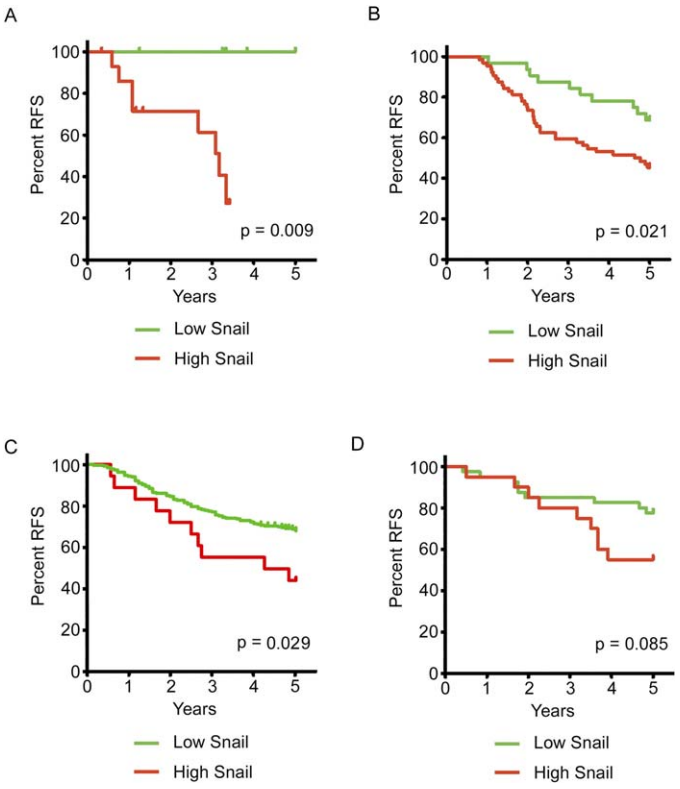

Figure 5. *Snail* expression predicts decreased relapse-free survival in women with breast cancer

Five year relapse-free survival for human tumors with low versus high *Snail* expression in microarray data sets representing the following. **A:** Locally advanced human breast cancers (Sorlie et al., 2003); **B:** lymph node-negative human breast cancers (van't Veer et al., 2002); **C:** lymph node-negative, ER-positive, and ER-negative human breast cancers (Wang et al., 2005); and **D:** hormone receptor-positive human breast cancers (Ma et al., 2004).

74 locally advanced ER-positive and ER-negative primary breast cancers from patients without distant metastases at the time of presentation (Sorlie et al., 2003). Microarrays containing probe sets capable of detecting Snail had been used in analyzing 22 of these 74 samples. Applying the Kaplan-Meier estimator to high and low Snail-expressing groups revealed a significant correlation between breast cancers expressing high levels of Snail and decreased relapse-free survival ($p = 0.009$) (Figure 5A).

A similar analysis of relapse-free survival was performed using microarray data derived from 97 ER-positive and ER-negative breast cancers that were lymph node-negative at presentation (van't Veer et al., 2002). Similar to locally advanced tumors, high Snail expression in node-negative human primary breast cancers was significantly associated with decreased distant relapse-free survival ($p = 0.021$) (Figure 5B). A third analysis of the relationship between Snail expression and the rate of distant relapse in 286 patients with lymph node-negative breast cancers (Wang et al., 2005) showed a similar significant association ($p = 0.029$) between breast cancers expressing high levels of Snail and decreased relapse-free survival (Figure 5C). Finally, we analyzed a fourth set of 60 hormone receptor-positive breast cancers from patients without distant metastases at the time of tumor resection (Ma et al., 2004). Once again, a trend between high Snail expression and rapid tumor recurrence was observed within this patient group, although the association did not reach statistical significance ($p = 0.085$) (Figure 5D).

Taken together, these data demonstrate that high levels of *Snail* expression in human primary breast cancers consistently predict decreased relapse-free survival. The demonstration that four different Snail probes on four different microarray platforms applied to four different patient sample sets representing 464 patients each yield the same result strongly suggests that the association between Snail expression and relapse-free survival is not specific to the particular probes used to detect Snail, the particular platforms on which the microarray studies were performed, or the particular characteristics of the patient populations that were represented in these studies. Moreover, since a significant association between *Snail* expression and rate of relapse was observed for both locally advanced and node-negative breast cancers, as well as among ER+ and ER– breast cancers, this analysis suggests that *Snail* expression may be an important prognostic indicator for breast cancer in a variety of clinical contexts.

The prognostic significance of *Snail* expression is comparable to classic prognostic indices

Having determined that *Snail* expression levels predict relapse-free survival in women with breast cancer, we wished to compare the magnitude of this association with currently used clinical prognostic markers. Accordingly, the Cox proportional hazards regression was used to calculate hazard ratios (HR) for relapse based on *Snail* expression, tumor size, tumor grade, lymph node status, ER status, and HER2 status (Table 1). This analysis revealed that the risk of breast cancer relapse associated with high levels of *Snail* expression (HR >2-fold) is comparable to those associated with currently used clinical prognostic indicators, including that of ER status, tumor grade, and lymph node status.

Snail predicts relapse-free survival independently of other prognostic markers

Since several prognostic markers have previously been established for human breast cancer, we asked whether the prognostic significance of *Snail* was attributable to its correlation with other markers of aggressive tumor behavior. Two characteristics of breast cancers in women that have been found to be strong predictors of relapse-free survival are tumor size at diagnosis and lymph node status (Carter et al., 1989; Valagussa et al., 1978). Therefore, we analyzed the association between *Snail* expression and tumor size by four methods: contingency table analysis of binned *Snail* expression levels versus binned tumor size; *Snail* expression level as a continuous variable versus binned tumor size; binned *Snail* expression versus tumor size as a continuous variable; and the correlation between *Snail* expression and tumor size, in which each is analyzed as a continuous variable. None of these methods revealed a significant correlation between *Snail* expression and tumor size in any of the three human breast cancer data sets analyzed (Table 2).

Similarly, we attempted to correlate *Snail* expression with lymph node status using both contingency table analysis and

Table 1. Hazard ratios for relapse based on clinical prognostic indicators

		Snail status	Tumor size	Tumor grade	LN status	ER status	HER2 status
Sorlie	HR	**	1.11	2.02*	1.13	2.59*	3.04*
	95% CI	**	0.49–0.51	1.13–3.59	0.59–3.03	1.16–5.79	1.04–8.86
	p value	**	0.810	0.017	0.494	0.021	0.042
van't Veer	HR	2.24*	1.73*	3.01*	N/A	2.32*	4.10*
	95% CI	1.11–4.53	1.19–2.53	1.60–5.68	N/A	1.28–4.19	1.72–9.80
	p value	0.025	0.004	0.001	N/A	0.005	0.002
Wang	HR	2.04*	N/A	N/A	N/A	1.18	1.06
	95% CI	1.06–3.92	N/A	N/A	N/A	0.76–1.85	0.63–1.80
	p value	0.034	N/A	N/A	N/A	0.460	0.820
Ma	HR	2.21	1.33	2.01	0.97	N/A	1.90
	95% CI	0.87–5.57	0.80–2.23	0.94–4.30	0.44–2.14	N/A	0.45–8.13
	p value	0.094	0.269	0.073	0.947	N/A	0.385

Hazard ratios, 95% confidence intervals, and p values for breast cancer relapse within 5 years of surgery, based on *Snail* expression grouping or previously described prognostic factors. Data are presented for each of the four human breast cancer data sets analyzed. HR, hazard ratio; CI, confidence interval; LN, lymph node. Statistically significant hazard ratios ($p < 0.05$) are indicated by asterisks. The hazard ratio is mathematically infinite, since no tumors with low Snail expression recurred. No confidence interval or p value can be generated.

Snail expression level as a continuous variable versus lymph node status. Again, neither of these methods revealed a significant correlation between *Snail* expression and lymph node status in either of the data sets analyzed (Table 2). These findings indicate that *Snail* expression predicts relapse-free survival independently of two of the most commonly used prognostic markers for breast cancer relapse.

We next examined the association between *Snail* expression and other prognostic indicators, including histological tumor grade, ER status, and *HER2/neu* expression. Using analytical methods similar to those described above, no consistent correlation between *Snail* expression and histological tumor grade was detected (Table 2). Similarly, a correlation between *Snail* expression and HER2 expression was observed in only one of 16 such analyses. We were, however, able to detect a statistically significant negative correlation between *Snail* expression and ER expression in two of the four data sets.

We then asked whether *Snail* expression might correlate with a specific cellular subtype of breast cancer associated with poor outcome. Sorlie et al. have previously described array-based methods for the molecular classification of human breast cancers into five subtypes: luminal A, luminal B + C, basal, ERBB2+, and normal breast-like (Sorlie et al., 2001, 2003). Of these, the basal and ERBB2+ subtypes have been

Table 2. p values for association between *Snail* expression and 5 year relapse-free survival or prognostic indicators

	Analysis	Sorlie	van't Veer	Wang	Ma
Relapse-free survival	Log-rank test based on *Snail* status	0.009*	0.021*	0.029*	0.085
Tumor size	*Snail* status versus size status	0.193	0.793	N/A	0.367
	Snail expression versus size status	0.397	0.686	N/A	0.533
	Snail status versus size	0.335	0.908	N/A	0.986
	Snail expression versus size	0.456	0.737	N/A	0.522
Tumor grade	*Snail* status versus grade status	0.104	0.454	N/A	0.644
	Snail expression versus grade status	0.010*	0.965	N/A	0.287
Lymph node status	*Snail* status and lymph node status	0.648	N/A	N/A	1.000
	Snail expression versus lymph node status	0.375	N/A	N/A	0.162
ER status	*Snail* status versus ER status	1.000	0.636	<0.0001*	N/A
	Snail expression versus ER status	0.601	0.901	0.0002*	N/A
	Snail status versus *ESR1* expression	0.438	0.518	<0.0001*	0.024*
	Snail expression versus *ESR1* expression	0.146	0.806	<0.0001*	0.001*
	Snail expression versus *ESR1* expression in ER+ tumors	0.166	0.258	0.0621	<0.001*
	Snail expression versus *ESR1* expression in ER– tumors	0.784	0.475	0.0584	N/A
HER2 status	*Snail* status versus HER2 status	1.000	0.660	0.7482	0.272
	Snail expression versus HER2 status	0.505	0.455	0.3850	0.095
	Snail status versus *HER2* expression	0.217	0.843	0.0353*	0.707
	Snail expression versus *HER2* expression	0.373	0.933	0.1387	0.467
Ductal versus lobular	*Snail* status versus histological status	1.000	N/A	N/A	1.000
	Snail expression versus histological status	0.293	N/A	N/A	0.994
Sorlie subtype classification	*Snail* status versus Sorlie subtype status	1.000	0.520	N/A	N/A
	Snail expression versus Sorlie subtype status	0.334	0.976	N/A	N/A

Correlation between *Snail* expression and 5 year relapse-free survival, and between *Snail* expression and previously described prognostic factors for breast cancer. p values are shown for each of the four human breast cancer data sets analyzed. "Status" designation refers to groupings of samples as described in the Experimental Procedures. All other designations refer to the variable as continuous, based either on microarray-determined expression levels or on size. Contingency table analyses (group versus group) were tested by Fisher's exact test. Group versus continuous variable analyses were tested by ANOVA. Correlations between two continuous variables were tested by the p value of the Pearson correlation coefficient. Asterisks indicate statistically significant correlations ($p < 0.05$).

Table 3. Proportional hazards analyses of *Snail* status and selected covariates

	van't Veer			Wang		
	HR	95% CI	p value	HR	95% CI	p value
Snail status	2.09*	1.03–4.24	0.040	N/A	N/A	N/A
Tumor grade	1.51*	1.51–5.33	0.001	N/A	N/A	N/A
Snail status	2.12*	1.05–4.29	0.037	N/A	N/A	N/A
Tumor size	1.70*	1.16–2.49	0.006	N/A	N/A	N/A
Snail status	2.18*	1.08–4.42	0.030	2.00	0.99–4.02	0.053
ER status	2.15*	1.18–3.94	0.013	1.04	0.65–1.67	0.876
Snail status	2.06*	1.01–4.19	0.046	2.07*	1.07–4.00	0.031
HER2 status	3.68*	1.53–8.86	0.004	1.12	0.66–1.89	0.687

Proportional hazard ratios, confidence intervals, and p values for 5 year relapse-free survival in the van't Veer et al. (2002) and Wang et al. (2005) data sets based on *Snail* expression groups controlled for tumor grade, tumor size, ER status, and HER2 status, and for each of these factors controlled for *Snail* expression. Asterisks indicate statistically significant hazard ratios ($p < 0.05$).

shown to be associated with the worst prognosis, whereas the luminal A subtype has been associated with the best prognosis, in both the Sorlie and van't Veer data sets. Neither contingency table analysis nor continuous variable analysis of *Snail* expression differences between these subtypes revealed any correlation between *Snail* expression and tumor subtype classification (Table 2). Similarly, for the Sorlie and Ma data sets, information was provided regarding whether tumors were of ductal or lobular origin. Again, *Snail* expression levels were not correlated with ductal versus lobular tumor type in either data set (Table 2). In aggregate, our results indicate that the ability of *Snail* expression levels to predict survival is not attributable to its preferential expression in a previously described aggressive breast cancer subtype.

Finally, although *Snail* expression did not correlate with most previously described prognostic markers, we examined whether adjusting for any of these factors would alter the ability of *Snail* expression to predict relapse-free survival. Notably, the association between *Snail* expression and decreased relapse-free survival remained statistically significant even when adjusted individually for tumor grade, tumor size, ER status, or HER2 status (Table 3). Therefore, although limited correlations were observed between *Snail* expression and either tumor grade or HER2 status in one data set each, and between Snail and ER status in two data sets, the correlation between elevated *Snail* expression and decreased relapse-free survival is not attributable to any of these associations. Overall, these findings demonstrate that *Snail* expression strongly predicts relapse-free survival in women with breast cancer and that this association is largely independent of previously described prognostic factors.

Discussion

Tumor recurrence is a cardinal manifestation of breast cancer progression and represents the principal cause of death from this disease. Preventing relapse by eradicating residual neoplastic cells will likely require simultaneous targeting of the multiple pathways that contribute to tumor survival and progression. Moreover, with the increasing use of molecularly targeted cancer therapies—many of which are cytostatic—the identification of molecular pathways that permit tumor cells to escape blockade of a dominant oncogenic pathway, survive in a latent state, and eventually reestablish malignant growth has become even more important. While many animal models developed over the past two decades have provided insight into the molecular events leading to tumor development, few have addressed the molecular and cellular events that lead to recurrent disease.

We now present in vivo genetic evidence for a molecular mechanism that contributes to mammary tumor recurrence. Our data demonstrate that *Snail* is spontaneously upregulated during the process of tumor recurrence in intact animals and that tumor recurrence is accompanied by EMT, a cellular transition that has been linked to breast cancer progression and in which Snail has been implicated. Consistent with a causal role for Snail in EMT and breast cancer progression, we have further shown that Snail is sufficient to induce EMT in primary tumor cells and to promote mammary tumor recurrence in vivo and that high levels of *Snail* expression strongly predict decreased relapse-free survival in women with breast cancer. These observations strongly implicate Snail in the process of breast cancer recurrence.

Although we identified Snail on the basis of its role in the recurrence of neu-induced mammary tumors in mice, we have also shown that the ability of Snail to predict relapse-free survival in women is not due to its preferential expression within any currently recognized aggressive subtype of breast cancer. Moreover, its prognostic significance is independent of the most robust currently recognized molecular and cellular markers. If Snail's role in human tumor recurrence were restricted to *HER2/neu*-amplified breast cancers, we would not have expected to observe a general relationship between *Snail* expression and breast cancer recurrence, since *HER2/neu*-amplified tumors represent only a small proportion of the cancers in the data sets examined. Consistent with a more general role for Snail in human breast cancer recurrence, we have demonstrated that *Snail* is not preferentially expressed in *HER2/neu*-amplified tumors or in other breast cancer subtypes. Since a significant association between *Snail* expression and the likelihood of relapse was observed for both ER-positive and ER-negative breast cancers, *HER2/neu*-amplified and unamplified breast cancers, and breast cancers with and without lymphatic

spread, our data suggest that Snail may be linked to breast cancer recurrence in a wide variety of clinical contexts.

Importantly, the association that we detected between *Snail* expression and disease relapse in humans was observed in primary tumors, implying that Snail activation within primary tumors may play a role in promoting tumor recurrence. Consistent with this, we have demonstrated that enforced Snail upregulation in murine primary tumors is sufficient to promote tumor recurrence. Moreover, in the small subset of primary neu-induced tumors that did not regress fully when neu was downregulated, we identified high levels of *Snail* expression comparable to those found in recurrent tumors (Figure 3B). Nevertheless, it is worth noting that the association between breast cancer relapse and Snail expression in primary human tumors is not inconsistent with our observation that spontaneous *Snail* upregulation in this mouse model occurs in recurrent tumors. First, *Snail* is expressed at detectable levels in primary neu-induced tumor cells in mice—albeit at lower levels than in recurrent tumors. Second, *Snail* expression levels in recurrent human breast cancers are at present unknown, since these tumors are generally unavailable for molecular analysis. As such, based upon our findings in mice, we predict that analysis of recurrent human breast cancers will reveal elevated levels of Snail expression compared to primary tumors.

The association between EMT and breast cancer progression has significant support within the literature, although no upstream regulator of both processes has previously been correlated with relapse-free survival. A subset of human breast cancers and human breast cancer cell lines display features of EMT (Hajra et al., 2002; Yang et al., 2004); in addition, suppression of CK8 and expression of vimentin have each been correlated with decreased survival of breast cancer patients (Fuchs et al., 2002; Niveditha and Bajaj, 2003). Moreover, loss of E-cadherin expression in breast cancers has been shown to be positively correlated with advanced histological grade, metastasis, and decreased disease-free as well as overall survival (Gamallo et al., 1993; Guriec et al., 1996; Heimann et al., 2000). Given the strong repression of E-cadherin observed in Snail-expressing tumor samples, the well-described association of E-cadherin with poor prognosis may in some cases reflect Snail activity. Indeed, an inverse association between *Snail* and *E-cadherin* expression has been reported in some human breast cancers (Zhou et al., 2004). Nevertheless, our data do not address which of Snail's many functions are most relevant to recurrence or the maintenance of residual disease.

Snail expression has previously been correlated with histological grade and lymph node metastasis in a panel of 17 human breast cancers (Blanco et al., 2002). While we were able to detect a significant correlation between *Snail* expression and histological grade in one breast cancer data set by one method of analysis, we did not find consistent correlations between *Snail* expression and histological grade across most data sets, nor between *Snail* expression and lymph node status in any of the data sets examined. As such, it is possible that in larger, more diverse tumor sets *Snail* expression levels may not correlate significantly with tumor grade or lymph node status, yet may remain a significant predictor of relapse-free survival.

Of note, we demonstrated that *Snail* expression does not consistently correlate with tumor size, HER2 status, ductal versus lobular subtype, or luminal versus basal subtype, and that its prognostic significance is comparable to currently used prognostic markers. This suggests that the association between Snail and relapse-free survival is not simply due to its selective expression in a presently recognized subset of aggressive breast cancers. Thus, independent of most commonly used prognostic indicators, high *Snail* expression identifies a subset of human breast cancer patients at high risk for recurrence. Moreover, the HR associated with increased Snail expression is comparable to that observed for ER status, which represents one of the most commonly used clinical prognostic variables as well as the most commonly used target for breast cancer prevention and treatment.

Notably, another molecule involved in mesoderm development, Twist, has been reported to be a positive regulator of *Snail* expression in *Drosophila*. However, in experiments implicating Twist in tumor metastasis, Yang et al. were unable to detect induction of *Snail* in human mammary epithelial cells induced to undergo EMT by forced Twist expression (Yang et al., 2004). We were also unable to detect a significant correlation between *Twist* expression and *Snail* expression in the Ma, Sorlie, or Wang human breast cancer data sets (*Twist* expression was not available for the van't Veer data set). This is in agreement with data suggesting that Snail and Twist function independently in mice (Carver et al., 2001; Soo et al., 2002). As such, the observed associations between Twist and metastasis and between Snail and recurrence are likely to represent independent manifestations of—and mechanisms for—mammary tumor progression.

It is important to note that the findings presented in this manuscript most directly address the process of local recurrence. Isolated local recurrence following breast conservation surgery with radiation treatment occurs in ~10%–20% of women at 10 years and typically comprises up to one-third of all recurrences (Doyle et al., 2001; Fisher et al., 1991; Fortin et al., 1999; Schmoor et al., 2000). As such, local recurrence is in and of itself a critical problem to understand. However, there are additional reasons to believe that the findings presented here may be relevant to distant as well as local recurrence. For example, a priori considerations alone would suggest that the processes by which tumor cells—whether local or distant—survive in a latent state and ultimately reestablish malignant growth are likely to be related mechanistically. Consistent with this presumption, multiple studies have demonstrated that local recurrence is strongly associated with an increased risk of both distant relapse (relative risk [RR] = 5.1) and mortality (RR = 3.6) (Doyle et al., 2001; Fisher et al., 1991; Fortin et al., 1999; Schmoor et al., 2000). Moreover, even the *timing* of local recurrences after surgery is similar to that of distant recurrences (Demicheli et al., 2004). Together, these observations suggest that local and distant recurrence may result from similar processes. Thus, beyond the recognition that local recurrence is itself important to understand, the associations between local and distant recurrence suggest that the mechanisms responsible for one may be informative for the other. As such, our findings that *Snail* promotes local recurrence in mice and predicts local and distant recurrence in humans raise the possibility that Snail may contribute to both processes.

Similarities between local and distant recurrences notwithstanding, distinctions between the processes of recurrence and metastasis must be considered since the mechanisms that allow latent tumor cells to survive and ultimately reestablish malignant growth are likely to differ from those that permit

actively growing tumor cells to invade and spread to distant sites. While metastasis is a sine qua non of distant spread, it is clearly not required for local recurrence. Moreover, even in the case of distant recurrence, the mechanisms that permit metastatic cells to remain in a quiescent state and reemerge at a later time are not the same as those that permit distant spread in the first place. As such, while the processes of recurrence and metastasis likely share a number of similar features, distinct mechanisms almost certainly contribute to each process. It is therefore essential to distinguish these processes experimentally to permit elucidation of these separate mechanisms.

Given the enormous number of women at risk for breast cancer recurrence, Snail's ability to predict relapse-free survival in women, coupled with evidence that Snail promotes the recurrence of mammary tumors in mice, suggest that further studies of this transcriptional repressor may be clinically relevant. Beyond the ability to identify women at high risk for relapse, our findings raise the important possibility that breast cancer recurrence might be prevented by therapeutic agents that inhibit Snail, either by preventing its upregulation or by inhibiting its function. For example, GSK-3β inhibits *Snail* transcription and also promotes its nuclear export and proteasome-dependent degradation by a phosphorylation-dependent mechanism (Zhou et al., 2004; Bachelder et al., 2005). Accordingly, inhibition of GSK-3β activity by the PI3K/AKT, MAPK, and Wnt signaling pathways promotes Snail stabilization and activation. In addition, phosphorylation of Snail by Pak1—which is activated by PI3K via AKT and the small GTPase Rac (Tang et al., 2000)—enhances Snail's repressor activity by promoting its nuclear accumulation (Yang et al., 2005). As such, the development of cancer therapeutics that target these upstream pathways may provide a dual mechanism for the inhibition of Snail activity both by activating GSK-3β and by inhibiting Pak1. Thus, while it is not possible to confirm a causal role for Snail in human breast cancer recurrence until drugs are available to inhibit this pathway, we hypothesize that treatment of patients with pharmacologic agents that block Snail expression or function may be effective for preventing breast cancer relapse. Snail may thereby represent an important new target for a generation of cancer therapeutics directed against specific molecules involved in breast cancer recurrence.

Experimental procedures

Animals, tissues, immunostaining, and molecular analyses

MMTV-rtTA/TetO-NeuNT mice were engineered, housed, induced with 2 mg/ml doxycycline, monitored for tumor development, and sacrificed as previously described (Gunther et al., 2002; Moody et al., 2002). Details for tissue fixation, immunofluorescence, immunohistochemistry, Northern blotting, and microarray analyses are provided in the Supplemental Experimental Procedures.

Tumor grafting and retroviral transduction

Details for culture of tumor cells harvested from chronically induced MTB/TAN mice, retroviral transduction, and tumor grafting are provided in the Supplemental Experimental Procedures.

Human breast cancer data sets

Descriptions of human breast cancer microarray data sets that were interrogated and methods used for data analysis, including survival analysis, calculation of HRs, and estimation of the association between *Snail* expression and established prognostic factors, are described in detail in the Supplemental Experimental Procedures.

Supplemental data

The Supplemental Data include Supplemental Experimental Procedures and can be found with this article online at http://www.cancercell.org/cgi/content/full/8/3/197/DC1/.

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